Grainyhead-like 3, a transcription factor identified in a microarray screen, promotes the specification of the superficial layer of the embryonic epidermis

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Abstract

The *Xenopus* ectoderm consists of two populations of cells, superficial polarised epithelial cells and deep, non epithelial cells. These two cells types differ in their developmental fate. In the neural ectoderm, primary neurons are derived only from the deep cells. In the epidermal ectoderm, superficial cells express high levels of differentiation markers, while most of the deep cells do not differentiate until later when they produce the stratified adult epidermis. However, few molecular differences are known between the deep and superficial cells. Here, we have undertaken a systematic approach to identify genes that show layer-restricted expression by microarray analysis of deep and superficial cells at the gastrula stage, followed by whole mount *in situ* hybridisation. We have identified 32 differentially expressed genes, of which 26 show higher expression in the superficial layer and 6 in the deep layer and describe their expression at the gastrula and neurula stage. One of the identified genes is the transcription factor Grhl3, which we found to be expressed in the superficial layer of the gastrula ectoderm and the neurula epidermis. By using markers identified in this work, we show that Grlh3 promotes superficial gene expression in the deep layer of the epidermis. Concomitantly, deep layer specific genes are switched off, showing that Grlh3 can promote deep cells to take on a superficial cell identity in the embryonic epidermis.

Introduction

The *Xenopus* embryonic ectoderm is a bilayered epithelium generated during the blastula stage of development by orientated cell divisions (Chalmers et al., 2003). These divisions generate outer superficial cells and underlying deep cells. The two cell types do not mix either before or during gastrulation (Keller, 1978), therefore the deep and superficial cells of the late blastula/gastrula embryo give rise directly to the deep and superficial cells of the bilayered post-gastrulation ectoderm. The superficial and deep cells of the ectoderm then show a number of differences in their fate.

In the neural ectoderm, where the superficial and deep cells are both incorporated into the neural tube only the deep cells give rise to the primary neurons (Hartenstein, 1989). The superficial cells give rise to what have been described as secondary precursors (Hartenstein, 1989) but their long-term fate is unknown. This difference in differentiation appears to be caused by the superficial cells having a low competence for neuronal differentiation (Chalmers et al., 2002). In turn, the low competence can be explained by the superficial cells expressing inhibitors of neuronal differentiation, such as ESR6e (Chalmers et al., 2002) and Geminin (Seo et al., 2005). However the molecular basis for the difference in differentiation and competence remains to be fully established.

Differences in the differentiation profile of the superficial and deep cells are also seen in the epidermal ectoderm. The outer epithelial cells express high levels of differentiation markers, keratin and the monoclonal antibody 2F7.c7, from an early stage (Jamrich et al., 1987; Jones and Woodland, 1986). In contrast most of the inner or basal cells do not differentiate until metamorphosis when they proliferate further to produce a stratified epidermis (Furlow et al., 1997; Nieuwkoop and Faber, 1967). At this stage the outer layer of cells is lost. The *Xenopus* embryonic epidermis is similar to the mammalian embryonic epidermis, which has an outer layer/periderm, overlying basal (stem) cells (Gilbert, 2003). The outer epidermal layer/periderm is shed during development and the basal cells give rise to the stratified epidermis. Therefore a key cell fate decision during development of the epidermis is the choice between periderm or basal cell fate. In *Xenopus* the two cell types show a number of other differences in

fate. Ciliated cells differentiate from some of the deep layer cells but then intercalate into the superficial cell layer (Deblandre et al., 1999; Drysdale and Elinson, 1992) and at the extreme anterior of the embryo the cement gland forms from the superficial cells (Wardle and Sive, 2003). Isolation experiments have shown that the two cell types behave differently when isolated from the pre gastrula embryo, separated from each other and cultured (Jones and Woodland, 1986). This demonstrates that the two cell types already have different levels of specification prior to the onset of gastrulation. However, the molecular basis for the difference in fate in the epidermal ectoderm, as with the neural, remains to be explained.

Deep and superficial ectodermal cells show a number of other differences in addition to their differences in cell fate. For example, they differ with regards to their contribution to wound healing. Although both layers are necessary for wound healing, only the superficial layer cells form actin purse strings (Davidson et al., 2002). Finally, the two cell types show differences in cell polarity (Muller and Hausen, 1995). The outer cells are epithelial cells where polarity is regulated by aPKC and Lgl2 (Chalmers et al., 2005b), while the deep cells are non epithelial. In summary the superficial and deep cells show differences which span many biological processes.

In order to understand how these differences are mediated it is important to identify the genes which are differentially expressed by the two cell types. To do this we have carried out a microarray screen, followed by an *in situ* screen, to identify genes with different expression between the superficial and deep cells. This identified 32 differentially expressed genes, 26 with superficial specific expression and 6 with deep specific expression. The genes identified code for a wide range of proteins including transmembrane proteins, transcription factors, signal transduction proteins, components of the cytoskeleton and a novel protein. These genes will be useful as molecular markers for the two cell types and are also likely to be involved in controlling their differentiation. We went on to investigate the function of one of the genes, the transcription factor Grainyhead-like 3 (Grhl3). We report that Grhl3 expression is expressed in the superficial layer of the ectoderm of the gastrula embryo and subsequently in the superficial layer of the epidermis. We then use marker genes identified in this study and cell lineage labelling to show that Grhl3 has the potential to drive the basal cells of the epidermal ectoderm to acquire a transcription profile

characteristic of superficial epidermal cells, thereby being capable of promoting superficial cell identity in the deep cells.

Results

A microarray and *in situ* screen

Superficial and deep cells were isolated from animal caps of late stage 9/early stage 10 embryos and used to make probes which were hybridised to a cDNA microarray containing 42,566 cDNA clones (Shin et al., 2005). This identified 193 differentially expressed cDNA clones, 150 with stronger expression in the superficial cells and 43 cDNAs with stronger expression in the deep cells. The microarray is not based on a unigene set and contains multiple cDNAs for some genes. The cDNA clones identified by the microarray screen corresponded to 98 unique genes, 68 in the superficial cells and 30 in the deep cells (supplementary data 1).

In order to eliminate false positives a second screen was then carried out. The 98 positives from the microarray were all checked by *in situ* hybridisation to see if they showed differential expression between the two cell types. This identified a total of 32 differentially expressed genes. Of these 26 had higher expression in the superficial cells (Table 1, Fig. 1-5) and 6 had higher expression in the deep cells (Table 2, Fig. 6). Blast searches were then carried out to identify the differentially expressed genes. The fold difference in expression from the microarray and the top blast hit for each cDNA clone is shown in Tables 1+2.

An overview of the differentially expressed genes

The differentially expressed genes encoded many different types of proteins, including transcription factors, transmembrane proteins, cytoskeletal molecules and signal transduction molecules (Table 1+2). The screen also identified one gene which appears to be novel. It has homologues in other species, including human and mouse, but no analysis beyond sequencing has been carried out. The predicted protein also

contains no conserved structural domains (Based on NCBI conserved domain search). We have called this gene, SG1, for **S**uperficial **G**ene one.

To our knowledge only 3 out of the 32 genes have been shown previously to be differentially expressed between the two cell types. These are epidermal keratin (Jamrich et al., 1987), XIRG (*Xenopus* Immune Response Gene), a protein of unknown function (Schmidt and Richter, 2000) and TA-2, a transmembrane protein (Table 3; also known as Xwig1, Klingbeil et al., 2001), all superficial layer specific genes. Therefore, the combined screens identified 29 new genes, which are differentially expressed between the two cell types.

Genes with superficial cell expression

The *in situ* hybridisation analysis was carried out at stage 10 (Fig. 1-5) and stage 14 (Fig. 1-5 and Table 3) and as mentioned above, 26 genes were found to be expressed in the superficial cells at stage 10 (Fig. 1-5). In addition, to superficial cell expression chemokine receptor 4 and XRnd1 (Wunnenberg-Stapleton et al., 1999) are also expressed in the involuting tissue at the marginal zone (Fig. 1+4).

The majority of genes that are superficially expressed at stage 10 (24 out of 26) had expression in the superficial layer of the epidermal ectoderm at stage 14, although in many cases not exclusively (Fig. 1-5 and Table 3). For example many genes which showed strong expression in the superficial layer of epidermal ectoderm also showed weaker expression in the deep epidermal ectoderm (e.g. Fig. 3, keratin type1 and Fig. 5, SG1) and superficial neural ectoderm (Fig. 3, uroplakin III). 12 out of the 26 genes showed some expression in the archenteron, and 2 of those, EFRIN and tRNA splicing endonuclease 54, are only expressed in the archenteron. Expression in the archenteron was not unexpected since it is also derived from the superficial layer of the blastula (Keller, 1975). Interestingly, expression in the superficial layer of the neural ectoderm at stage 14 was quite rate, with only 5 genes (poliovirus receptor related 2, calponin H3, alsin, putative Phytanoyl-CoA dioxygenase and XRnd1) showing expression and none of the genes showing exclusive expression in this tissue.

Genes with deep cell expression

The expression of the 6 deep cell specific genes is shown in Fig. 6 and Table 4. These include an extracellular matrix enzyme (hyaluronan synthase 1), the nuclear factor

(prothymosin alpha), the transcription factor cofactor (vestigial like 4) and a transcription factor (Sox11). As with the superficial cell specific genes, the expression pattern of these genes is relative simple at stage 10 but tends to evolve into a more complicated one at stage 14. Out of the 6 genes, 5 showed some expression in the deep layer of the epidermal ectoderm (with the exception being Sox11 which was expressed in both neural layers). Unlike the superficial genes, expression in neural tissue was relatively common, with 4 out of the 6 genes found in neural tissue at stage 14. The expression of 3 of these was restricted to deep layer of neural tissue (neurofilament triplet H protein, prothymosin alpha and FK-506 binding protein 4; Table 4) while Sox11 was expressed by both layers.

Overall, this combined screen greatly increases the repertoire of genes showing differential expression by the superficial and deep cells. This will be useful for future studies into the different characteristics of these two cell types in at least two ways. Firstly they can be used as layer-specific markers. Secondly the genes may represent regulatory molecules involved is specifying the differences seen between the two cells types. We go on to investigate one such molecule the transcription factor, Grhl3.

Grhl3 can promote deep epidermal cells to express superficial cell markers

One of the superficial specific genes was found to be a member of the Grainyhead (Grh) family of transcription factors. In vertebrates there are known to be 3 members of the family, called Grainyhead-like (Grhl) 1-3 (Huang and Miller, 2000; Kudryavtseva et al., 2003; Ting et al., 2003b; Wilanowski et al., 2002). The gene isolated in our screen is most closely related to Grhl3.

In *Xenopus,* Grhl3 is expressed in the superficial cells at stage 10 and then in the superficial layer of the epidermal ectoderm and in the lining of the archenteron at stage 14 (Fig. 1). As a transcription factor it has the potential to mediate the differences seen between the superficial and deep cells. Grhl3 was overexpressed and the effect on the epidermis analysed using markers for superficial and deep cells identified in this study. At a morphological level Grhl3 caused a thickening of the deep layer of the epidermis (Fig 7, the boundaries of the epidermis are marked by black bars). When the expression of the superficial markers, keratin type 1 epidermal, claudin 4 and XRnd1, was examined all were found to show strong expansion of their normal expression domains (Fig. 7E-G). A third superficial marker, XIRG was also

found ectopically, but in this case the effect was limited to a few scattered cells (Fig.7H). These findings suggest that Grhl3 can expand the population of cells that are expressing superficial cell markers.

Grhl3 causes the down regulation of deep/progenitor specific genes.

Many genes with strong expression in the superficial cells, such as XIRG and keratin have weaker expression in the deep cells. One could argue that the thickening of the deep layer of cells caused by Grhl3 overexpression makes normal levels of expression more apparent. Alternatively, the cells in the expanded domain may be superficial cells in terms of their expression profile. In the second case, the cells should not express deep cell specific genes. To distinguish between these possibilities we overexpressed Grhl3 and scored the effect on the deep cell markers (Fig. 7M-P). Grhl3 was found to down regulate the expression of the deep cell markers hyaluronan synthase 1 and prothymosin alpha (Fig. 7M+N). The amount of down regulation varied between cells with some cells having no deep marker expression, some with intermediate amounts and some with high levels of expression. Interestingly the groups are clustered together which produces the apparent holes (Fig. 7M+N arrow), which are groups of cells with very low levels of deep cell marker expression.

The deep cells are not a uniform population as they contain some differentiating cell types. These include ciliated cells which differentiate from the deep cells of the epidermis (Deblandre et al., 1999) and primary neurons which differentiate from the deep cells of the neural ectoderm (Hartenstein, 1989). Grhl3 expression was found to inhibit α tubulin, a marker of ciliated cells, and N tubulin, a marker of neurons (Fig. 8F). Therefore, Grhl3 can cause an expansion of superficial specific markers and inhibit the expression of deep specific markers.

Grhl3 causes the deep cells to take on a superficial cell fate

The expansion of superficial cell gene expression and inhibition of deep cell gene expression could be caused by Grhl3 reprogramming the deep cells to take on a superficial cell fate. However, it could also be caused by superficial cells translocating into the deep layer, among the original deep cells. To find out whether this latter possibility is true we biotin-labelled the outer membrane of the egg, just prior to RNA injection. This outer membrane is partitioned by division and forms the apical membrane of all superficial cells in the gastrula and early neurula embryo. This

method has been previously used to follow the fate of superficial cells during gastrulation (Minsuk and Keller, 1996) and endoderm development (Chalmers and Slack, 2000). The advantage of this method is that ingression of outer cells to the inner layer at any stage between the time of grh injection (2-cell stage) and the time of analysis (early neurula) would be detected. Embryos were labelled with biotin before the first cleavage, injected with Grhl3+LacZ or GFP+LacZ and then analysed at neural plate stages (Fig. 8A-C). In control injected embryos, biotin label was localised to the apical membrane of the outer cells; there were no biotin-labelled internal cells because normally, there is no translocation of outer cells to the inner layer. In Grhl3 injected embryos the lacZ marker was used to find the Grhl3 injected region, and the area that had been thickened by Grhl3 misexpression located. In these Grhl3 thickened regions biotin labelling was also restricted to the outer cells, as in the controls (Fig. 8B). Therefore, there were no ectopic biotin-labelled cells in the inner layers of the epidermis. It is worth noting that simultaneous in situ hybridisation was not done as the chromogenic signal could have masked any potential biotin signal in the deep cells. In summary Grhl3 causes an expansion of superficial cell markers and an inhibition of deep cell markers in the deep cell layer. This occurs without endogenous superficial cells translocating into the deep layer. Thus, Grhl3 is reprogramming the endogenous deep cells to take on a superficial cell fate.

Grhl3 causes a thickening of the epidermis but does not increase cell proliferation

Finally we addressed the question of what is causing the thickened epidermis. One possibility is that Grhl3 is promoting proliferation in a fashion similar to FoxG1, which thickens the epidermis and promotes proliferation (Hardcastle and Papalopulu, 2000). To address whether Grhl3 can promote proliferation, dividing cells in control and Grhl3 injected embryos were labelled with 5-Bromo-2'-Deoxy-uridine (BrdU). The total number of cells per section and the percentage of these that were proliferating was then counted (Fig. 8D-I). Grhl3 produced a significant increase ($P<0.05$) in the average number of cells per section in the epidermis (Fig. 8H). However it did not produce a significant increase in the percentage of cells proliferating. The epidermis normally shows low levels of proliferation (Saka and Smith, 2001), a finding that is confirmed by our BrdU experiment. Therefore, we additionally examined whether Grhl3 can promote proliferation in the neural ectoderm, a tissue which shows higher levels of proliferation at this stage (Saka and

Smith, 2001). Similar to the epidermis, Grhl3 did not promote proliferation in the neural ectoderm. Therefore, it seems likely that Grhl3 is causing a thickening of the ectoderm by a method other than promoting proliferation (discussed below).

Discussion

Differences between the superficial and deep cells of the early embryonic ectoderm in *Xenopus* embryos, are important for many aspects of epidermal and neuronal differentiation. We are interested in understanding the molecular basis of these differences and in this work we identified 32 differentially expressed genes. These will provide useful markers for future studies. The genes include transmembrane proteins, secreted proteins, signal transduction proteins, a novel protein and transcription factors and so are also likely to include proteins with key roles in the development and differentiation of these cells. Indeed, we show that one of these differentially expressed genes, the transcription factor Grhl3 can promote superficial epidermal fate in the deep cells of the ectoderm.

Cell fate specification within the developing epidermis

In *Xenopus* BMP signalling acts to promote epidermal cell fate (Wilson and Hemmati-Brivanlou, 1995) by activating a number of transcription factors, such as Msx1 and Xvent2 (Onichtchouk et al., 1996; Suzuki et al., 1997). These genes are direct targets of BMP signalling and simultaneously act to promote epidermal cell fate and inhibit neural cell fate. There are also a number of transcription factors such as XAP-2 that lie further down the cascade (Luo et al., 2002). These promote epidermal cell fate but do not inhibit neural cell fate. Therefore there is a well established cascade that leads to specification of an epidermal cell fate. However, within the epidermis cells do not all follow the same fate. The embryonic epidermis contains a superficial protective layer and an inner layer containing the stem cells which give rise to the adult skin (Furlow et al., 1997; Nieuwkoop and Faber, 1967). Therefore the choice between a superficial or a deep cell fate represents a crucial part of early

epidermal development. In this study keratin type 1 epidermal, claudin4, XRnd1 and XIRG were used as markers for superficial cells, while hyaluronan synthase 1 and prothymosin alpha were used as markers for the deep cells. These markers make it possible to investigate how differences in fate are generated between the superficial and deep cell layers of the epidermis.

Grhl3 can promote deep epidermal cells to take on superficial cell identity

One of the superficial specific genes was found to be a member of the Grainyhead family of transcription factors. This family get their name from the *drosophila* transcription factor Grainyhead (also called Elf-1 and NTF-1; Bray et al., 1989; Bray and Kafatos, 1991; Dynlacht et al., 1989). The phenotype of the *Drosophila* mutant has multiple defects including a defective epidermal cuticle (Bray and Kafatos, 1991; Nusslein-Volhard et al., 1984), and defective epidermal repair (Mace et al., 2005) demonstrating that Grainyhead is required for correct epidermal differentiation. This role in epidermal development appears to have been evolutionarily conserved as knocking down the *C elegans* Grainyhead gene causes defects in the cuticle of the worm (Venkatesan et al., 2003).

In vertebrates there are 3 genes that are closely related to *Drosophila* Grainyhead, called Grainyhead-like (Grhl) 1-3 (Huang and Miller, 2000; Kudryavtseva et al., 2003; Ting et al., 2003b; Wilanowski et al., 2002). These have previously been called MGR, LBP-32, GET-1, SOM and BOM. There are also more divergent members of the Grainyhead family, such as LBP-1a, which are more closely related to *Drosophila* CP2 than *Drosophila* Grainyhead (Wilanowski et al., 2002). Recent work has begun to show a role for the Grhl genes in vertebrate epidermal development. In *Xenopus,* Grhl1 is expressed in the epidermis and its expression is positively regulated by BMP signalling. Loss of function experiments showed that knocking down Grhl1 leads to defects in the epidermis and loss of epidermal keratin expression (Tao et al., 2005). In the mouse knocking out Grhl3 causes spina bifida, loss of the epidermal permeability barrier and reduced expression of the epidermal gene Tgase1 (Ting et al., 2005; Ting et al., 2003a).

The effect of Grainyhead gain of function has not been addressed in the intact vertebrate epidermis. Therefore, in this work we asked if the Grhl3 is sufficient to promote cell fate changes within the context of an intact epidermis. Our results show

for the first time that Grhl3 is sufficient to promote superficial layer-specific gene expression in the basal cells, which are normally set aside as precursor cells that differentiate later in development. The decision between periderm or basal cell (superficial and deep in *Xenopus*) is the first cell fate decision that is made during epidermal development. This work shows that Grhl3 is able to promote cells to follow one of these two paths and take on superficial cell identity.

Grainyhead and the cell cycle

Grhl3 overexpression also caused a thickening of the epidermis. A strong prediction from this observation could be that Grhl3 promotes proliferation in the ectoderm. This would be consistent with recent findings in *Drosophila* where knocking out Grhl3 alters rates of cell proliferation in the nervous system (Almeida and Bray, 2005; Cenci and Gould, 2005). However we failed to detect any change in proliferation after Grhl3 overexpression, either in the epidermal or neural ectoderm. Interestingly unlike Grh loss of function, Grh overexpression in *Drosophila* also did not affect proliferation (Almeida and Bray, 2005). This suggests that the thickening is caused by another mechanism. Prior to gastrulation there are several layers of deep cells which undergo radial intercalation to produce a single layer (Keller, 1980). The ciliated cells, a subpopulation of the deep cells, also intercalate among outer cells (Deblandre et al., 1999; Drysdale and Elinson, 1992). If either or both of these behaviours were inhibited, the deep layer of the epidermis would be thickened. Thus, one possible cause of the thickening is that Grhl3 alters the morphogenetic behaviour of the deep cells, perhaps as a consequence of their transformation to superficial-like cells. Although the mechanistic details remain to be elucidated in future work, a morphogenetic effect is a promising possibility since known targets of Grh include proteins involved in cell adhesion and cell polarity (this work; Almeida and Bray, 2005; Lee and Adler, 2004) and Grh is required for tracheal morphogenesis in Drosophila (Hemphala et al., 2003).

Experimental procedures

Microarray screen

The microarrays used contained 42,566 PCR products which were amplified from cDNAs and spotted onto two slides (Shin et al., 2005). Superficial and deep layer cells were isolated from late stage 9/early stage 10 embryos (Fig. 1). At this stage, but not before, it is relatively easy to manually separate the two layers. This was done cutting the intact animal cap and placing them in calcium magnesium free medium (88 mM NaCl, 1mM KCL, 2.4 mM $NaHCO_3$, 7.5mM Tris, PH 7.6). The superficial layer was peeled off and collected. The deep cells were then removed and placed in a separate tube. Batches of 30 of each cell layer were snap frozen on dry ice and stored at -80 °C. This was carried out over several days until enough layers were collected. Total RNA was isolated from these batches and combined. Probes were synthesised as described previously without RNA or cDNA amplification (Shin et al., 2005; Tran et al., 2002). 40 μg of total RNA was used for each labelling reaction. The hybridisations were carried out twice for each slide, once with each dye combination (e.g. superficial Cy3+deep Cy5 and superficial Cy5+ deep Cy3) so included a dye swap. This made a total of 4 hybridisations using 160 μg of total RNA and 1950 superficial and deep cell layers. The hybridised slides were scanned and the data imported into Acuity as described (Chalmers et al., 2005a). Once in Acuity the cDNAs which were at least 2 fold different between the two samples in both hybridisations, were identified for each of the two slides and imported into Excel. The duplicate clones which corresponded to the same gene were then identified using sequence available at the XDB3 website (http://xenopus.nibb.ac.jp/) and our own sequencing if required. The genes were then identified by analysing the sequence of the cluster with blastn and blastx. In the paper we often refer to the gene name but the corresponding clone used for the *in situs* is shown (Table 1+2). For one of these genes there was no clear homologue except for unnamed sequences. We named this gene SG1 (accession number).

***In situ* screen**

One clone was then selected for each of the 98 genes (supplementary data) and ordered from the XDB website (kindly proved by the NIBB/NIG/NBRP *Xenopus laevis* EST project). The chosen clone corresponded to the most 5' clone in the cluster, ensuring the clone was long as long as possible. This means the clones should make good *in situ* probes and are also most likely to be full length for future overexpression experiments. The unique EST identifier of the original and 5' clone

data is shown (supplementary data 1). The clones were mini prepped, PCR amplified using T3 and T7 primers to generate templates, which were used to produce dig labelled *in situ* probes. *In situs* were then carried out for each clone at stage 10 and stage 14 using standard procedures, except the stage 10 embryos (and some stage 14) were cut in half during fixation prior to *in situ* hybridisation to reduce probe trapping in the blastocoel. This also ensures the superficial and deep cells have equal exposure to the probe. Where required the embryos were embedded using gelatin albumen and sectioned on a vibratome as previously described (Chalmers et al., 2002). Future requests for the clones used in this project should be made to the NIBB EST project, via the XDB3 website (http://xenopus.nibb.ac.jp/).

Overexpression experiments

At the time of carrying out the experiments there was not a full length Grhl3 clone in the *Xenopus laevis* EST data base so we identified a full length clone (TGas117i15, accession number CR848623) from the *Xenopus tropicalis* EST data base (Gilchrist et al., 2004). The coding sequence amplified by PCR and cloned into the Stu1 site of CS2. LacZ pCS2 and GFP3 pCS2 were also used as previously described (Chalmers et al., 2005b; Chalmers et al., 2002). RNA for injection was made using the message machine kit (Ambion) and the RNA injected into 1 cell of the 2 cell embryo. 1ng of Grhl3 (or 0.5 ng depending on the batch of RNA) or 1/0.5ng of GFP was injected combined with 0.5ng of LacZ as a lineage label. Embryos were then fixed in MEMFA at stage 14, processed for X-Gal staining and *in situ* hybridisation using standard procedures. The following were used as superficial markers; keratin type 1 epidermal (Xl075f19), claudin 4 (Xl011e03), XRnd1 (Xl071h23) and XIRG (Xl095m11) and the following as deep markers; hyaluronan synthase 1 (Xl109a23), prothymosin alpha (Xl069j18), alpha tubulin 26e9.1 (Pollet et al., 2003) and N Tubulin (Oschwald et al., 1991). Embryos were then sectioned as above.

Biotin labelling

Albino embryos were labelled with active biotin essentially as previously described (Minsuk and Keller, 1997). Embryos were dejelleyed 30 minutes after fertilisation and placed in 10 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in 0.1x MMR for 30 minutes. They were then washed in 10 mM glycine and injected with RNA as described above. At neural plate stages the vitelline membrane was removed, they were fixed for 2 hours in MEMFA, processed for X-Gal staining, embedded in wax, sectioned and the

biotin visualised using alkaline phosphatase conjugated streptavidin (Vector labs) as described (Minsuk and Keller, 1997).

BrdU labelling

BrdU labelling was carried out using the BrdU labelling and detection kit 1 (Roche, 11 296 736 001). Grhl3+GFP or GFP injected neural plate stage embryos were injected 3x with 10 nl of the neat BrdU labelling reagent, incubated for 1 hour, fixed in MEMFA for one hour and stored at -20°C in MeOH. The embryos were sectioned on a cryostat using the fish gelatin protocol (Fagotto and Gumbiner, 1994). The BrdU was then visualised using the supplied anti BrdU antibody diluted 1/100 with incubation buffer and an anti mouse 568 secondary antibody (1/1000, molecular probes). The nuclei were stained with TOPRO3 (1/5000 added to the secondary antibody, Molecular Probes). This made it possible to count the number of cells per section and the % of these that were dividing cells. The injected area was identified using the GFP fluorescence. The counts were based on the following numbers of cells and sections, GFP epidermal 210/12; GFP neural 267/12; Grhl3 epidermal 236/9; Grhl3 neural 245/9.

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Figure Legends

Figure 1. A microarray and *in situ* screen to isolate genes differentially expressed between the superficial and deep cells. **A**, superficial and deep cells were isolated from early *Xenopus* embryos and used to make a probes which were hybridised to the microarray. The positives were then screened by *in situ* hybridisation to confirm that they showed differential expression. B, Genes with superficial expression; Part 1. A wholemount and a section are shown for each gene at gastrula (left hand panels) and neural plate stage (right hand panels). The gastrula stage wholemounts have the animal hemisphere to the top. The sections show expression in the animal hemisphere. The chemokine receptor 4 wholemount embryo is bisected to show the expression in the involuting marginal zone. The stage 14 wholemounts are shown as lateral (L) or dorsal views (D) as indicated, with anterior to the left. The stage 14 sections are transverse and show the expression in the epidermis, except for the krupple like factor where the expression at the border of the neural plate is shown. The poliovirus receptor related 2 has expression in the deep epidermis (arrow head) as well as the superficial cell epidermis. Arrows indicate areas of expression. The genes are labelled with the top blast hit and unique EST identifier (e.g. Xl075n14).

Figure 2. Genes with superficial expression; Part 2. A wholemount and a section are shown for each gene at gastrula (left hand panels) and neural plate stage (right hand panels). The gastrula stage wholemounts have the animal hemisphere to the top. The sections show expression in the animal hemisphere except for NEPH1 which shows the marginal zone as it has stronger expression in the marginal zone. The stage 14 wholemounts are shown as lateral (L) or dorsal views (D) as indicated with anterior to the left. The stage 14 sections are transverse and show expression in the epidermis except for NEPH1 which is sagital. NEPH1 has a complicated expression pattern which includes the notochord (white arrow), two bands in the anterior neural plate (black arrows) and endoderm (arrow head). Arrows indicate areas of expression. The genes are labelled with the top blast hit and unique EST identifier (e.g. Xl088m05).

Figure 3. Genes with superficial expression; Part 3. A wholemount and a section are shown for each gene at gastrula (left hand panels) and neural plate stage (right hand panels). The gastrula stage wholemounts have the animal hemisphere to the top.

The sections show expression in the animal hemisphere. The stage 14 wholemounts are shown as lateral (L) or dorsal views (D) as indicated with anterior to the left. The stage 14 sections are transverse and show expression in the epidermis. Arrows indicate areas of expression. The genes are labelled with the top blast hit and unique EST identifier (e.g. Xl085e06).

Figure 4. Genes with superficial expression; Part 4. A wholemount and a section are shown for each gene at gastrula (left hand panels) and neural plate stage (right hand panels). The gastrula stage wholemounts have the animal hemisphere to the top. The sections show expression in the animal hemisphere. The stage 14 wholemounts are shown as lateral (L) or dorsal views (D) as indicated with anterior to the left. The stage 14 sections are transverse and show expression in the epidermis except for alsin and EFRIN. Alsin has expression in the neural (arrow head) and epidermal (arrow) superficial ectoderm. The only expression shown by EFRIN was some very weak expression in the archenteron shown by a sagital section. Arrows indicate areas of expression. The genes are labelled with the top blast hit and unique EST identifier (e.g. Xl092a09).

Figure 5. Genes with superficial expression; Part 5. A wholemount and a section are shown for each gene at gastrula (left hand panels) and neural plate stage (right hand panels). The gastrula stage wholemounts have the animal hemisphere to the top. The sections show expression in the animal hemisphere. The stage 14 wholemounts are shown as lateral (L) or dorsal views (D) as indicated with anterior to the left. The stage 14 sections are transverse and show expression in the epidermis except for tRNA splicing endonuclease 54 which is sagital and shows some weak expression in the archenteron. Arrows indicate areas of expression. The genes are labelled with the top blast hit and unique EST identifier (e.g. Xl044m03).

Figure 6. Genes with deep expression. A wholemount and a section are shown for each gene at gastrula (left hand panels) and neural plate stage (right hand panels). The gastrula stage wholemounts have the animal hemisphere to the top. The sections show expression in the animal hemisphere. The stage 14 wholemounts are shown as frontal (F) or lateral (L) views as indicated. Lateral views have with anterior to the left and frontal veiws dorsal to the top. The stage 14 sections are transverse and show expression in the epidermis except for Sox11 and Neurofilament triplet H protein.

Sox11 is sagital while Neurofilament triplet H protein is transverse, both have expression in the neural ectoderm. Arrows indicate areas of expression. The genes are labelled with the top blast hit and unique EST identifier (e.g. Xl067c17).

Figure 7. Grhl3 can expand the expression of superficial markers and inhibit the expression of deep cell markers. Embryos were injected with GFP (A-D and I-L) or Grhl3 (E-H and M-P) and LacZ as a lineage marker. Embryos were fixed at neural plate stages, stained for B galactosidase (green) and for the *in situ* marker (purple), as indicated. The sections are transverse and show the epidermal ectoderm except for N tubulin which shows the neural ectoderm. Grhl3 but not GFP control injections caused an expansion of the superficial cell markers (Fig. 7E-H). An exception was XIRG which unlike the other superficial cell markers showed a very weak response to Grhl3 which was limited to a few cells (arrows). Grhl3 also caused an inhibition of the deep cell markers (Fig. 7M-P). The cells with strong reduction in expression clustered together producing apparent holes/bubbles in the epidermis (Fig. 7M+N, arrows). Grhl3 caused a thickening of the epidermis, the boundaries of the epidermis are highlighted with black bars.

Figure 8. Grhl3 does not cause cells to leave the superficial layer and migrate into the deep layer and it does not promote proliferation. A-C, Grhl3 does not cause cells to leave the superficial layer. Superficial cells were labelled with biotin and injected with GFP+LacZ or Grhl3+LacZ, as indicated. Negative control embryos were not stained with biotin or injected. The embryos were then fixed at neural plate stages, stained for B galactosidase (green) and for biotin (purple). A transverse section of the epidermis is shown for each treatment which shows that biotin labelled superficial cells remained on the outside as the epidermis was expanded. Arrows highlight biotin staining and black bars indicate the boundaries of the epidermis. D-I, Grh does not promote proliferation. Embryos were injected with GFP or Grhl3+GFP, then at neural plate stages incubated with BrdU for 1 hour and fixed. The embryos were then stained for BrdU incorporation (red). The injected area was visualised using the injected GFP (green) and a nuclear stain (blue) was used to count the number of cells. Transverse sections of the neural and epidermal ectoderm are shown. Arrows highlight BrdU positive cells. The epidermal ectoderm has very low levels of proliferation, the cells highlighted by arrows are under the ectoderm in the mesoderm or endoderm (This can be seen by comparing them to the localisation of the GFP). H-

I, Quantification of the BrdU result. Grhl3 caused a significant increase ($P<0.05$) in the number of cells seen per section in the epidermis (H), consistent with the thickening seen previously, but did not produce a significant increase in the % of cells proliferating (I). This is based on at least nine sections and 210 cells for each treatment and area.

transcription factors			
XL075n14	6.4	Grainyhead like 3	Grainyhead family transcription factor
XL068o23	2.8	blood island enriched kruppel like factor	zinc finger transcription factor
secreted molecules			
XL096m11	9.5	apelin precursor	secreted neuropeptide
Transmembrane			
XL005k12	7.8	chemokine receptor 4	G protein coupled receptor
XL093g24	2.3	poliovirus receptor related 2	Immunoglobin family transmembrane receptor
XL088m05	27	NEPH1	Immunoglobin single pass trans membrane
XL024g17	4.9	ER to nucleus signaling 1	transmembrane protein kinase
XL045a16	3.8	monocarboxylate transporter 4	Immunoglobin transmembrane protein
XL152n04	7.1	TA-2	transmembrane protein
XL108f19	5.7	uroplakin II	apical transmembrane protein
XL085e06	2.3	uroplakin III	apical transmembrane protein
XL011e03	17.4	claudin 4	tight junction protein
Cytoskeletal			
XL075f19	3.8	keratin, type I, epidermal,	intermediate filament
XL022l23	3.2	keratin, type II cytoskeletal	intermediate filament
XL107b16	3.6	epiplakin 1	cytoplasmic linker protein
XL092a09	2.6	calponin H3	cytoskeletal binding protein
signal transduction			
XL071h23	4.2	XRnd1	small GTPase
XL055h20	3.4	alsin	guanine nucleotide exchange factor
other			
XL038l06	6.7	carcinoma associated protein-like	unknown
XL095m11	4.9	XIRG	unknown
XL033k18	3.1	EFRIN	rab11 interacting protein
XL044m03	3.7	possible E3 ubiquitin ligase	possible E3 ubiquitin ligase
XL010a16	4.1	putative phytanoyl-CoA dioxygenase (PhyH) enzyme	Possible peroxisomal enzyme
XL100n18	2.3	guanylate kinase 1	guanylate kinase
XL019o09	10	SG1	unknown
XL041l24	2.6	tRNA splicing endonuclease 54	Splicing enzyme

Table 1 Superficial cell specific genes. The columns are, from the left, clone, average fold difference, top blast hit and protein type.

transcription factors

clone	fold difference	top blast hit	protein type
XL067c17	2.6	Sox11	HMG box transcription factor

transmembrane

clone	fold difference	top blast hit	protein type
XL109a23	2.9	hyaluronan synthase 1	membrane hyaluronan synthase

cytoskeletal

clone		top blast hit	protein type
XL038h23	2.0	neurofilament triplet H protein	intermediate filament

other

clone	fold difference	top blast hit	protein type
XL069f20	3.8	FK506-binding protein 4	peptidyl-prolyl cis trans isomerase
XL069j18	2.9	prothymosin alpha	small acidic nuclear protein
XL078j17	3.9	vestigial like 4	transcription factor co factor

Table 2 deep cell specific genes

clone	top blast hit	st14 expression
XL075n14	Grainyhead like 3	superficial epidermal ectoderm and lining of archenteron
XL068o23	blood island enriched kruppel like factor	border of neural plate (both layers), cement gland, and weaker in epidermal superficial tissue
XL096m11	apelin precursor	Superficial epidermal ectoderm and lining of archenteron
XL005k12	chemokine receptor 4	Superficial epidermal ectoderm
XL093g24	poliovirus receptor related 2	Neural superficial ectoderm and epidermal superficial and deep ectoderm with a gap of reduced expression at the edge of the neural plate. Archenteron lining
XL088m05	NEPH1	notochord, two stripes in anterior neural plate, lining of archenteron
XL024g17	ER to nucleus signaling 1	Superficial epidermal ectoderm
XL045a16	monocarboxylate transporter 4	Superficial epidermal ectoderm
XL152n04	TA-2	Superficial epidermal ectoderm. Also small part of the posterior archenteron
XL108f19	uroplakin II	Superficial epidermal ectoderm
XL085e06	uroplakin III	Superficial epidermal and weaker superficial neural ectoderm
XL011e03	claudin 4	Superficial epidermal ectoderm and posterior archenteron
XL075f19	keratin, type I, epidermal,	Superficial epidermal ectoderm
XL022l23	keratin, type II cytoskeletal	Superficial epidermal ectoderm
XL107b16	epiplakin 1	Superficial epidermal ectoderm+notochord
XL092a09	calponin H3	neural and epidermal superficial ectoderm. Also notochord
XL071h23	XRnd1	neural and epidermal superficial ectoderm. Muscle and anterior archenteron lining
XL055h20	alsin	neural and epidermal superficial ectoderm
XL038l06	carcinoma associated protein-like	Superficial epidermal ectoderm and archenteron lining
XL095m11	XIRG	Superficial epidermal ectoderm and posterior archenteron
XL033k18	EFRIN	Weak in the archenteron
XL044m03	possible E3 ubiquitin ligase	epidermal superficial ectoderm and both layers of anterior neural ectoderm
XL010a16	putative phytanoyl-CoA dioxygenase (PhyH) enzyme	neural and epidermal superficial ectoderm. Dorsal archenteron lining
XL100n18	guanylate kinase 1	Superficial epidermal ectoderm
XL019o09	SG1	Superficial epidermal ectoderm
XL041l24	tRNA splicing endonuclease 54	weak in the floor of archenteron

Table 3. Expression of the superficial genes at the neural plate stage. Groups with similar expression are highlighted, the epidermal ectoderm in red, neural and epidermal in blue and archenteron in green. This does not mean they are only expressed in this tissue. For example epidermal superficial genes often have weaker expression in the neural ectoderm and deep epidermal cells. Four genes are not highlighted as they have complex patterns and do not fall into one of the groups.

clone	top blast hit	protein type
XL067c17	Sox11	both layers of neural ectoderm-broad pattern including trigeminal
XL109a23	hyaluronan synthase 1	deep layer of epidermal ectoderm and ventral mesoderm and endoderm
XL038h23	neurofilament triplet H protein	neural and epidermal deep ectoderm with weaker staining in superficial ectoderm
XL069f20	FK506-binding protein 4	deep neural and epidermal ectoderm, weaker in superficial ectoderm and endoderm
XL069j18	prothymosin alpha	deep neural with weaker deep epidermal ectoderm
XL078j17	vestigial like 4	deep epidermal ectoderm.

Table 4 Expression of deep cell specific genes in neural plate stage embryos

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Supplementary files

Supplementary file 1- microarray results for genes greater than 2 fold differentially expressed and corresponding IDs of the microarray and *in situ* clones.